

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A01N 1/02, C12P 19/34, C12M 3/00	A1	(11) International Publication Number: WO 99/52356 (43) International Publication Date: 21 October 1999 (21.10.99)
(21) International Application Number: PCT/US99/07816 (22) International Filing Date: 9 April 1999 (09.04.99) (30) Priority Data: 09/058,619 9 April 1998 (09.04.98) US (63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application US 09/058,619 (CIP) Filed on 9 April 1998 (09.04.98) (71) Applicant (for all designated States except US): CHARLOTTE-MECKLENBERG HOSPITAL AUTHORITY [US/US]; P.O. Box 32861, Charlotte, NC 28232 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): HALBERSTADT, Craig, R. [US/US]; 18708 Nautical Drive #3, Cornelius, NC 28031 (US). HOLDER, Walter, D., Jr. [US/US]; 4715 Cambridge Crescent Drive, Charlotte, NC 28226 (US). (74) Agents: POSORSKE, Laurence, H. et al.; Baker & Botts, L.L.P., The Warner, 1299 Pennsylvania Avenue, N.W., Washington, DC 20004 (US).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>
(54) Title: CREATION OF THREE-DIMENSIONAL TISSUES (57) Abstract A method of providing a vascularized, three-dimensional tissue in a living subject is disclosed. The method includes the steps of (a) creating, from a biocompatible material capable of supporting cell adhesion, growth, and migration, a porous construct containing cells to be transplanted, and (b) delivering the construct into an area of interest in the living subject to form a vascularized three-dimensional tissue. The preferred construct has a dimension in which it is about 50 μm to about 500 μm from the outermost surface to the center of the construct. The preferred construct also has an interconnected porous structure having a pore size of from about 10 μm to no greater than 300 μm . The cells within the preferred construct are no greater than 250 μm from an outer surface of the construct.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

CREATION OF THREE-DIMENSIONAL TISSUES

FIELD OF THE INVENTION

5 This invention relates to methods of tissue engineering, and particularly to a method of creating a vascularized three-dimensional tissue in mammal.

BACKGROUND OF THE INVENTION

10 Despite advances in medical technology, tissue loss remains a serious problem. Tissue damage can be caused by various reasons such as surgical removal of cancerous tissues and trauma. After the removal of the damaged tissue, reconstruction is often desired. Another reason relates to organ transplantation where it is possible to replace a damaged organ with a healthy donor organ. However, the organ transplantation approach has drawbacks. First, organs for
15 transplantation are scarce and one may have to wait for a long time before a suitable organ becomes available. Second, the immunological incompatibility between the donor and donee tissues limits the ability of the transplanted organ to survive and function, and further aggravates the availability problem. Moreover, the transplantation process itself is complex, invasive, and costly.

20 In contrast, a great number of cells can be obtained directly from the patient. These autologous cells can further be propagated in cell culture media. Moreover, the use of autologous cells obviates the tissue incompatibility problem. Thus, viable three-dimensional tissues, generated from isolated cells, are valuable for use in repairing tissue damages.

25 Current approaches for generating three-dimensional tissues *in vitro* from isolated cells have not been very successful. Extensive research has been conducted to develop techniques for transplanting cells directly into body tissues and developing a three-dimensional tissue from these cells *in vivo*. Cells seeded within three-dimensional support constructs such as enclosed encapsulation membranes or porous
30 matrices have been transplanted along with the constructs and rendered to survive,

grow, and function *in vivo*. (See, e.g., U.S. Patent Nos. 4,353,888; 4,487,758; and 4,902,295.) However, there has been little success with respect to the formation of three-dimensional tissues, especially vascularized three-dimensional tissues, from transplanted cells.

5 Transplanted cells require nutrients, growth factors, hormones as well as oxygen for survival. The transplanting techniques used heretofore generally do not provide an effective mechanism to support the transplanted cells. This is especially problematic in the formation of vascularized tissues. These techniques are unable to establish adequate vascularized beds that provide necessary nutrients to
10 the transplanted cells for the formation of three-dimensional tissues.

Furthermore, the approaches heretofore known generally require invasive surgical procedures for delivering cells into body tissues. For example, U.S. Patent No. 5,716,404 discloses a method for reconstruction or augmentation of breast tissue by implanting isolated cells with a polymeric matrix. The polymeric
15 fibrous matrix used therein has a structure similar to the silicone implants now used. Complex and invasive surgery has to be performed to insert the matrix into breast tissue.

A major challenge in mammalian cell transplantation and developing vascularized three-dimensional tissue is promoting long-term cell survival of the
20 transplanted cells. The current approaches rely on transplanting cells on surfaces that have diffusional distances greater than 250 μm for nutrients which are provided by the surrounding host vasculature. Unfortunately, a necrotic core of tissue develops within the construct because of an inadequate supply of nutrients and gases. Therefore, there is need for an effective and substantially non-invasive
25 method of creating vascularized three-dimensional tissue *in vivo*.

SUMMARY OF THE INVENTION

Another object of the present invention is to provide a method for growing vascularized tissue in a human.

Another object of the present invention is to provide a method to deliver discrete tissue constructs containing cells into an area *in vivo*.

The discrete tissue construct acts as a functional unit, e.g., the growth of the transplanted cells and the vascular ingrowth of the cells from the surrounding tissue forms small cellular "islands", interconnected with the surrounding vasculature. As a result, a vascularized three-dimensional tissue is formed.

Thus, the invention is directed to methods for providing a vascularized three-dimensional tissue in which transplanted cells are no greater than 250 μm away from a nutrient source, i.e., a developing vascular bed. A porous construct containing cells for transplantation is delivered into an area of interest in a living subject where it is desired to build a vascularized three-dimensional tissue. The implanted porous construct supports the adhesion, growth and migration of the transplanted cells as well as the ingrowth of the tissue cells surrounding the construct, thus forming a three dimensional discrete tissue. Preferably, the delivery of the porous construct is performed in a non invasive manner, for example, by injection, or by delivering in a biocompatible pouch having an arteriole and venule connection from the outer pouch into the inner pouch. It is also preferred that a plurality of the porous constructs are delivered.

The porous construct is made from at least one biocompatible material which supports cell adhesion, growth, and migration. Such biocompatible materials can be bioresorbable or non-resorbable. The cells to be transplanted can be seeded in the porous construct. The porous construct has a dimension in which the distance is about 50 μm to about 500 μm from at least one point on the outer surface to the center of the construct, such that when cells are seeded within the construct, the innermost cell within the construct is no greater than about 250 μm away from an outer surface of the construct. In an average tissue, the maximum distance found between a metabolically active cell and a capillary is approximately 250 μm . This distance has been established *in vivo* due to diffusional limitations of oxygen and nutrients from the capillary to the surface of the cell. The construct also has

interconnected pores having a pore size of from about 10 μm to about 300 μm . The porous construct may also contain a signal (e.g., a growth factor or an extracellular matrix protein) for modifying, preferably promoting cell adhesion, growth, or migration.

5 In one embodiment of the invention, the porous construct containing cells to be transplanted is delivered directly to the area of interest. In another embodiment of the invention, a vascular bed is formed in the area of interest by implanting, before the delivery of the porous construct, a second construct which promotes the formation of a vascularized bed *in vivo*. The porous construct
10 containing cells to be transplanted is then delivered onto the vascularized bed for formation of a three-dimensional tissue.

 The method of this invention can be used in the reconstruction of many different tissues, such as breast tissue, pancreatic tissue, liver tissue, neural tissue, kidney tissue, muscle tissue and skin tissue. The present invention provides a
15 minimally invasive procedure and effective method for development of three-dimensional tissues.

BRIEF DESCRIPTION OF THE DRAWINGS

 The foregoing and other advantages and features of the invention, and
20 the manner in which the same are accomplished, will become more readily apparent upon consideration of the following detailed description of the invention taken in conjunction with the accompanying examples, which illustrate preferred and exemplary embodiments.

 Figure 1A shows a trichrome stain of alg-RGD (1X concentration of
25 RGD) beads implanted in the subcutaneous space of a rat and harvested after one week of implantation illustrating tissue ingrowth between the beads (40X magnification);

 Figure 1B shows a formation of a vascular bed at the interface of alg-RGD beads and tissue (200X magnification);

Figure 2A shows tissue ingrowth between the beads (trichrome stain of alg-RGD (100X concentration of RGD)) implanted in the subcutaneous space of a rat, harvested after two weeks of implantation (40X magnification);

Figure 2B shows the formation of a vascular bed at the interface of the beads and the tissue (200X magnification);

Figure 3 shows Factor VIII localization in tissue that has grown between alginate-RGD beads;

Figure 4 illustrates cross sections of collagen beads that were seeded with smooth muscle cells;

Figure 5A shows a trichrome stain of histological section (40X and 200X) of collagen beads harvested from a rat that had been implanted for 2 weeks;

Figure 5B illustrates two Factor VIII stains from the same section as Figure 5A indicating that vascular endothelial cells are located throughout the collagen bead implant. (Top picture is 40X magnification and bottom picture is 200X magnification); and

Figure 6 illustrates the growth of aortic smooth muscle cells implanted in collagen beads harvested after two weeks (400X magnification); and

Figure 7 illustrates the cell growth of endothelial cells stained for Factor VIII on a collagen bead harvested after two weeks.

Figure 8 is a bar graph comparing histological scoring of 2-week subcutaneous implants of alginate rods or beads or collagen beads.

Figure 9 is a bar graph comparing histological scoring of alginate-RGD bead subcutaneous implants at 2, 4 and 8 weeks from implant.

Figure 10 is a bar graph comparing area of alginate-RGD bead implants at various time intervals.

Figure 11 is a bar graph comparing width of alginate-RGD bead implant at different times.

Figure 12 is a bar graph comparing thickness of Alginate-RGD bead implants at different times.

Figure 13 is a bar graph comparing thickness of the capsule surrounding alginate-RGD bead implants at different times.

DETAILED DESCRIPTION OF THE INVENTION

5 This invention provides a method for developing vascularized three-dimensional tissue in a living subject such as a human. In one embodiment of this method, a porous construct seeded with cells for transplantation is created and is delivered into an area of interest in a living subject where it is desired to build a vascularized three-dimensional tissue. The implanted porous construct supports the
10 adhesion, growth and migration of the transplanted cells as well as the ingrowth of the tissue cells surrounding the construct, thus forming a three-dimensional discrete tissue.

The porous construct of this invention can be in any three-dimensional shape, so long as it has a dimension in which the distance between an
15 outer surface of the construct and the center of the construct is from about 10 μm to about 500 μm , preferably from about 25 μm to about 400 μm , more preferably from about 50 μm to about 250 μm . For example, the porous construct can be in the shape of spheres, particle beads, rods, triangles, threads, and cubes. In its most preferred form, the construct has a dimension such that the distance from each and
20 every surface to the center of the construct has a length of about 250 μm . The construct is configured so that the innermost cell situated in the center of the construct is no greater than 250 μm from at least one surface of the construct. In this manner, it becomes more probable that, after the construct having cells therein is delivered to an area of interest in a living subject, all the cells within the construct
25 are no greater than 250 μm from a nutrient source, e.g., a vascularized bed.

The distance into the construct at which cells are found is determined primarily by the presence of nutrients and oxygen which diffuse into the porous construct to nourish the cells. The maximum diffusion distance will depend in part on cell type and metabolic state, as well as the porosity of the construct, and may be

up to about 400 μm , or more typically 250-300 μm . For highly metabolic cells, the maximum diffusion distance may be about 150 μm . For particles with linear dimensions larger than the maximum diffusion distance, cells will only populate the portion of the particle which may be reached by diffusion of oxygen and nutrients.

5 Areas of the particle that are further from the surface than the maximum diffusion distance will be substantially free of cells. For particles with irregular shapes, the area populated by cells may include all portions of the particle which are within the maximum diffusion distance of at least one surface of the particle. This may include diffusion from the surface of macropores in macroporous particles.

10 The porous construct has an interconnected macroporous structure formed by one or more biocompatible materials which constitutes the skeleton of the structure. There should be a plurality of macropores on the surfaces of the construct connecting the macropores within the construct so that exchange of molecules and cell migration can occur between inside of the construct and the surrounding
15 environment. The diameter of the pores ranges from about 10 μm to about 300 μm , preferably from about 25 μm to about 200 μm , more preferably from about 50 μm to about 100 μm .

In particular, macroporous constructs have pores of sufficient dimension to permit the passage of cells by migration through the pores. For macroporous
20 constructs, vascular ingrowth proceeds into the large diameter pores, as well as the spaces between discrete constructs. Thus, the vascular bed formed by the ingrowth can provide nutrients and oxygen to cells within particles having overall diameters much larger than the 250 μm suggested by the diffusion in microporous spherical particles. The preferred constructs of the present invention are microscopic
25 particulates with macroporous structure. A plurality of these particles forms a mass with a abundance of channels both within and between particles, through which nutrient-containing medium (plasma, lymph, cell culture medium, etc.) may reach cells in the particles and through which vascular ingrowth may occur. The overall dimensions of these preferred particulates are typically on the order of 1 millimeter,

although particle sizes greater or smaller than this are also contemplated for the discrete, macroporous constructs of this invention.

By "biocompatible" is intended that the material used for making the porous construct does not substantially adversely affect any desired characteristics of the cells to be seeded within the construct and of the cells and tissues in the area of a living subject where the construct is to be delivered. It is also intended that the material used does not cause any substantially medically undesirable effect in any other area of the living subject. The materials are chosen such that the porous construct supports the adhesion, growth, or migration of cells. Thus, the biocompatible material used must allow cell adhesion, growth, and migration. Preferably, the biocompatible material should also be mechanically strong enough to support cells and to substantially maintain a dimension. Generally, the methods for testing a material's biocompatibility and mechanical strength is well known in the art. The biocompatible material may be synthetic or natural.

The biocompatible material may be a material that is non-resorbable such that it will remain once delivered inside the body of a living subject. More preferably the biocompatible material is bioresorbable and will be gradually degraded after the porous construct is delivered to an area of interest inside the body of a living subject. Such degradation should be in a slow and gradual fashion and preferably should take place after sufficient cells have grown within the construct to form an organized three-dimensional tissue. In this manner, the degradation of the bioresorbable material does not interfere with the formation of a three-dimensional tissue. Preferably, the bioresorbable material degrades within about 1 to 3 months after transplantation.

Examples of suitable bioresorbable materials include but are not limited to polyesters, polyethylene glycols, and hydrogels. Examples of suitable non-resorbable materials include but not limited to polytetrafluoroethylenes (Teflon), nylon, polycarbonates, and polyethylenes. Suitable examples also include but not limited to polysaccharides such as dextran, dextrin, starch, cellulose, agarose,

carrageenan, alginate (a carboxylated seaweed polysaccharide), and the like; synthetic polymers such as polyvinyl alcohols, polyacrylamides, polyamides, polyacrylates, polyesters, polymethacrylates, polyurethanes, polyphosphazene, copolymers of lactic acid and glycolic acid, copolymers of lysine and lactic acid, 5 copolymers of lysine RGD and lactic acid, and the like. Proteins such as collagens, copolymers of collagen and chondroitin sulfate (a proteoglycan component), and the like can also be used.

The construct may also contain a signal for modifying cell adhesion, growth, or migration, preferably stimulating or promoting the adhesion, growth, or 10 migration of the desired cells, and/or inhibiting the adhesion, growth, or migration of the undesired cells. The signals may be selected from growth factors, hormones, extracellular matrix proteins and other cellular adhesion peptides identified in the extracellular matrix protein. Growth factors include for example, epithelial growth factor (EGF), acidic or basic fibroblast growth factor (FGF), vascular endothelial 15 growth factor (VEGF), hepatocyte growth factor (HGF), heparin binding growth factor (HGBF), transforming growth factor (TGF), nerve growth factor (NGF), muscle morphogenic factor (MMP), and platelet derived growth factor. Examples of extracellular matrix proteins include fibronectin, collagens, laminins, and vitronectins; and the tri-peptide RGD (arginine-glycine-aspartate) that is found in 20 many of the extracellular matrix proteins. A signal can also be included to induce the ingrowth of the desired cells, e.g., smooth muscle cells and epithelial cells, surrounding the construct in the area within the body of a living subject. Preferably, compounds which inhibit undesired cells, such as cancerous cells and inflammatory cells, can also be included. The signals can be covalently linked to a biocompatible 25 material in the porous construct or associated with the porous construct by affinity or linked to a material that can be covalently linked to or associated by affinity with a biocompatible material in the construct. See, e.g., U.S. Pat. Nos. 4,517,686; 4,792,525; and 5,330,911, incorporated herein by reference.

The method of providing the porous constructs has been successfully applied to the development of many different three-dimensional tissues in mammals. The selection of the cells contained in the porous construct for delivery into an area of interest in the body of a living subject depends on the location and the type of
5 three-dimensional tissue desired. For example, if a three-dimensional pancreatic tissue is desired, the porous construct may contain pancreatic cells. In another example, if the development of constructs to replace absent breast tissue is wanted, cells such as vascular endothelial cells and smooth muscle cells can be used. Other cells, such as mesenchymal cells which include fibroblasts, chondrocytes, and
10 adipocytes can also be used alone or in conjunction with endothelial or smooth muscle cells. Other cell types that may be used in this invention include hepatocytes, renal tubular cells, Sertoli cells, thyroid cells, islet cells, skeletal muscle cells, neural cells, cardiac muscle cells, osteocytes, stem cells, and the like.

Various techniques for isolating cells from suitable sources are
15 generally known in the art. The cells used in this invention are preferably autologous, i.e., obtained from the living subject itself. The cells can also be allogeneic, i.e., obtained from a subject of same species as the subject of interest or xenogeneic, i.e., from a subject of different species. In addition, the cells can also be treated *in vivo* or *in vitro*, and before or after being incorporated into the porous
20 construct. For example, the cells may be cultured to expand in number or modified to change one or more characteristics. For example, it is generally known in the art to genetically modify cells *in vitro* by introducing a desired gene or by replacing an undesired gene with a desired one, thus improving the characteristics of the cells. Methods are also known in the art to modify the immunological character of
25 allogeneic or xenogeneic cells so that the cells are not substantially rejected by the host tissue when they are delivered to the area of interest. Immunologically inert cells, such as stem cells and embryonic cells, are preferably used to avoid immunological incompatibility.

The cells to be incorporated in the porous construct of the invention can be homogeneous cells from one source, or from different sources. Thus, heterogenous cells are used, the porous construct may have two or more biocompatible materials and two or more signals discussed above, each supporting a different type of cell in the construct. The number of cells to be incorporated into the porous construct can range from 10 cells/cm² to 1×10^8 cells/cm².

The porous construct can be made in many methods known in the art for making a porous scaffold supporting cell growth, with certain modifications of these methods to obtain the distinct features of the porous construct of the present invention as specified above. Such modifications will be apparent to an ordinarily skilled person in the art apprised of the present disclosure.

The macroporous lattice structure of the porous construct is formed during the process of polymerization of one or more polymers using conventional methods for making macroporous structures. Alternatively, the macropores in the construct are formed by dissolution of a polymer or removal of one material after polymerization. In another method, the construct is formed from a porous matrix of polymers in which the pores are formed by dissolution of one of the polymers. In that method, two polymerization precursors, a matrix polymer precursor (e.g., collagen, fibrin, etc.) and a reversible gel polymer precursor (e.g., alginate, gums, agarose, etc.) are polymerized in an aqueous solution to form a shape-retaining solid matrix comprising viable cells, matrix polymer and reversible gel polymer. The reversible gel polymer is then dissolved and removed to form an insoluble, porous matrix containing viable cells. This methodology is used to construct small discrete tissue constructs as described herein with the requisite size constraints and the appropriate macroporous materials. An alternative method is described in U.S. provisional application entitled: "Methods of Preparing Porous Hydrogel Materials and Products of the Methods", filed on even date herewith under Attorney Docket No. UMICH 8VI, and incorporated herein by reference.

The porous construct is made by any known technique of producing small beads or other discrete structures. Suitable techniques include but are not limited to pressure or air shear spraying, extrusion, emulsification, and droplet formation techniques such as electrostatic droplet formation, droplet formation by gravity, droplet formation by centrifugal forces, droplet formation using Raleigh liquid jet instability techniques, and droplet formation using inertial forces. For example, suitable porous constructs are prepared from collagen materials by solidifying a collagen solution or dispersion into dry beads using any known drying techniques including but not limited to spray drying and freeze drying. Also, beads having larger sizes are reduced to small sizes by, for example, grinding.

For example, for the development of autologous breast tissue, smooth muscle cells can be seeded and grown on porous polyglycolide beads or other porous biodegradable or naturally occurring materials (such as porous type I collagen beads). Once the smooth muscle cells reach a specified density, a coating of a hydrogel, for example an alginate RGD containing vascular endothelial cells is polymerized on the beads. The alginate with endothelial cells encompasses the beads, and intercalates within the beads. This provides a close contact between the endothelial cells and the smooth muscle cells causing the necessary cell communication to occur. The smooth muscle beads provide the physical structure necessary to create this tissue.

The porous construct should be sterilized before use. Examples of such techniques include but are not limited to UV irradiation, gamma irradiation, e-beam sterilization and sterilization using chemicals such as ethylene oxide. The sterilization method used must not substantially adversely affect the structure of the porous construct and its ability to support the formation of a vascularized three-dimensional tissue.

It is also preferred to coat the porous construct with materials that promote cell adhesion and attachment to the structure of the porous construct. Examples of such materials include but not limited to fibronectin, vitronectin,

collagens, polylysine, laminins, polypeptides derived from these extra-cellular matrices, and other cell adhesion molecules. Such coating can be done at any time, for example, coating on polymer precursors, coating of a prepared construct before seeding cells *in vitro*, or after seeding *in vitro* but before delivering the construct into
5 the body of a living subject of interest. An example of coating is to use porous beads such as porous polyglycolide beads and porous collagen beads, prepared as described above and coated with a hydrogel, for example, an alginate RGD, by polymerizing the hydrogel onto the beads. The hydrogel not only encompasses the beads, but also intercalates within the beads.

10 The cells to be transplanted with the porous construct can be incorporated therein while the porous construct is formed, for example, during the process of polymerization. Methods of incorporating cells during the formation of a porous structure are well known in the art. Alternatively, the cells can be seeded into a pre-formed porous construct. Since the pores of the porous construct are fairly
15 large as described above, cell seeding can be easily done, for example, by immersing the porous construct for a period of time in a cell culture medium having cells to be transplanted floating therein. The cell density in the medium and the period of time can be easily controlled to allow a desired number of cells to attach within the porous construct.

20 Optionally, the cells in the porous construct are allowed to grow *in vitro* for a period of time before they are transplanted along with the porous construct into the body of a subject of interest. Methods for doing so will be apparent to those skilled in the art. For example, a porous construct with cells attached therein can be conveniently immersed in a suitable culture medium. To
25 promote the growth and differentiation of the cells, suitable signal molecules which modify cell adhesion, growth, and migration, such as growth factors and extracellular matrix proteins, are added to the culture medium.

Once delivered to an area inside the body of a subject of interest, the porous construct also allows the cells in the area surrounding the construct to migrate into the construct, promoting the formation of a three-dimensional tissue.

In accordance with the present invention, at least one porous
5 construct having cells therein is delivered into an area inside the body of a living subject where a three-dimensional tissue is desired to be built. A plurality of porous constructs from 0.1 ml of volume to 500 ml of volume may be placed in the body. Each such discrete construct will develop and become interconnected with the surrounding vasculature and with other developed constructs, mimicking the
10 functional units in natural tissue, thus facilitating the development of a three-dimensional tissue.

The delivery of the porous constructs is preferably performed in a substantially non-invasive manner, avoiding any complex surgical procedures. For example, the constructs could be fashioned to be easily injected into the tissue of
15 interest. More than one injection may be desired. Alternatively, these constructs are delivered in a biocompatible pouch that has an arteriole and venule connection from the outer pouch into the inner pouch.

In one particular embodiment, a second construct is also used which may or may not contain cells. Once delivered into an area in the body of a living
20 subject of interest, this second construct is capable of stimulating the formation of a vascularized bed surrounding it. Once the vascularized bed is formed, the porous construct as described above having therein cells to be transplanted is delivered onto or near the vascularized bed. The pre-developed vascularized bed can better supply the necessary nutrients required by the transplanted cells.

25 Preferably, a porous construct as described herein is used as the second construct for pre-formation of a vascularized bed. However, so long as it can induce the formation of a vascularized bed, the second construct can be made from any biocompatible material and in virtually any shape or dimension, and is not limited by the features of the porous construct. Preferably, small spheres of about 5

µm are used to help create the vascularized tissue. In addition, the second construct need not be macroporous. Preferably, the second construct contains signals which stimulate the formation of a vascularized bed. An example of such signal is the tri-peptide arginine-glycine-aspartate (RGD). This tri-peptide can be optimally linked
5 to the second construct.

An example of such second construct is solid sodium alginate-RGD macro-beads of about 1 to 2 mm in diameter. These beads were shown to be capable of inducing the development of a vascularized bed inside the body of a living subject in a relatively short period. The second construct may be delivered by the same
10 methods used for the delivery of the porous construct as described above.

The present invention can be used in many different applications in mammals, particularly in human. Many types of vascularized tissues can be developed using the method of this invention, including but not limited to three-dimensional breast tissue, pancreatic tissue, liver tissue, kidney tissue, muscle
15 tissue, skin tissue, etc.

This method can be used in reconstructive therapy, reconstructing a body part that is injured or from which undesired tissue has been removed. In particular, the method of this invention may be used for reconstruction or repair of soft tissue defects. Typically, a plurality of discrete, macroporous constructs seeded
20 with cells selected from chondrocytes, fibroblasts, endothelial cells, smooth muscle cells, etc., will be implanted to replace missing soft tissue, forming a tissue mass to fill a depression or produce a protrusion, or to otherwise provide a bulky mass of tissue where it is needed. For example, using the method of this invention, a soft vascularized breast tissue can be built in the area where cancerous breast tissue has
25 been removed, thereby reconstructing the breast. To do so, porous constructs containing both normal vascular endothelial cells and smooth muscle cells isolated from the patient can be created and delivered to the pocket of the expanded breast tissue in accordance with the present invention.

Further, the method of the present invention can also be used to treat certain diseases in mammals.

For example, to treat insulin-deficient diabetes, three-dimensional pancreatic islets which release a desirable level of insulin can be transferred into
5 relatively vascular sites such as the subcutaneous space and in combination with the creation of a three dimensional vascular bed using these discrete tissue constructs.

In summary, it has been provided an effective and non-invasive method of creating a vascularized three-dimensional tissue in an area of interest in a mammal such as humans. A plurality of macroporous constructs are constructed
10 from biocompatible materials and contain cells to be transplanted for the development of the three-dimensional tissue. The porous constructs are delivered into the desired area in a substantially non-invasive manner. The dimension of porous constructs and the size of their macropores are made such that the transplanted cells are no greater than 250 μm apart from a nutrient source in the
15 body. As a result, adequate support is provided for the adhesion, growth and migration of the transplanted cells as well as the ingrowth of the tissue cells surrounding the construct, thus forming a three-dimensional discrete tissue.

The present invention is further illustrated by the following non-limiting examples. This invention may, however, be embodied in many different
20 forms and should not be construed as limited to the embodiments set forth herein; rather, this embodiment is provided so that this disclosure will be thorough and complete and will fully convey the scope of the invention to those skilled in the art.

EXAMPLE 1 Formation of A Vascularized Bed Induced by Sodium
Alginate RGD Macro beads.

Sodium alginate covalently coupled with arginine, glycine and
5 aspartic acid tri-peptide (RGD) (alg-RGD) was mixed in calcium-free, magnesium
free phosphate buffered saline (Gibco/BRL) at a concentration of 2 gm/100 mL
(2%).. After thorough mixing the alg-RGD was filtered sterilized using a 0.45 µm
filter (Nalgene unit). The alg-RGD solution was then aseptically dropped into a
sterile filtered solution of 1.5% calcium chloride solution (1.5 µm of calcium
10 chloride mixed with 100 µm of Milipore reverse osmosis water) using a syringe
pump set at a flow rate of approximately 50 mL/hr. Beads of approximately 1 to 2
mm in diameter were formed during this process. A subcutaneous pocket was then
created on the back of a Fisher Rat (female) by using a purse string suturing
technique (a 50 mL conical tube cap was used to mark the 39 mm circumference of
15 the suturing plane, a 4.0 prolene suture was used to follow the outline of the circle,
and the skin in the center of the circle was picked up using forceps), and one mL of a
bead slurry was transplanted into this subcutaneous space.

Figure 1A shows the histological results of a trichrome stain after one
week of implantation. Figure 1B shows the formation of a vascular bed at the
20 interface of the beads and the tissue that forms. The results indicate that a
vasculature can develop in a relatively short time period and may be induced by the
shape of the materials. However, this material was not designed to be macroporous
and did not provide a means for cellular in growth into the tissue construct. When
larger solid implants were used in a similar animal model, a capsule was formed
25 around the entire perimeter of the implant without the formation of a vascular bed
that would support cellular survival.

EXAMPLE 2 Formation of A Vascularized Bed Induced by Sodium Alginate-RGD Macro-beads.

Sodium alginate covalently coupled with arginine, glycine and aspartic acid tripeptide (RGD)(alg-RGD) (100X more concentrated than Example 1) was mixed in 0.8 g NaCl, 0.2g Na-hexametaphosphate in 100 mL of reverse osmosis water at a concentration of 1.2 gm/100 mL (1.2%). After thorough mixing, the alg-RGD was filter sterilized using a 0.22 μ m filter. The alg-RGD solution was then aseptically dropped into a sterile-filtered solution of 1.5% calcium chloride solution (1.5 gm of calcium chloride mixed with 100 Ml of reverse osmosis water) using a syringe pump set at a flow rate of approximately 50 mL/hour. Beads of approximately 1 to 2 mm in diameter were formed during this process. A subcutaneous pocket was then created on the back of a Fisher Rat (female) by using a purse string suturing technique (a 50 mL conical tube cap was used to mark the 39 mm circumference of the suturing plane, a 4.0 prolene suture was used to follow the outline of the circle, and the skin in the center of the circle was picked up using forceps), and one mL of a bead slurry was transplanted into this subcutaneous space. Figures 2A and 2B show the histological results after two weeks of implantation. Figure 3 is histological section stained for Factor VIII which is a protein that is located on vascular endothelial cells. The material was implanted in a purse string subcutaneous space and harvested after two weeks. The dark open circles in the tissue have been positively identified as vascular endothelial cells.

EXAMPLE 3 Creation of A Vascular Three-dimensional Tissue with a Porous Construct Made from Collagen.

The previous examples indicated that the transplantation of bead structures of an appropriate material would provide the impetus for vascular

ingrowth into an avascular, subcutaneous pocket. However, these materials were not porous and the size of the beads were approximately 1 to 2 mm in diameter.

To determine if a macroporous bead would enhance vascular ingrowth into these structures, type I collagen (bovine collagen) microspheres purchased from Cellex, Inc., Minneapolis, MN was transplanted. The beads, ranging in size from 400 to 700 μm in diameter and having a nominal pore size of 50 to 100 μm in size, were designed to entrap and grow mammalian cells *in vitro* for the production of biologics. A subcutaneous pocket was created on the back of a Fisher Rat (female) by using a purse string suturing technique (a 50 mL conical tubecap was used to mark the 39 mm circumference of the suturing plane, a 4.0 prolene suture was used to follow the outline of the circle, and the skin in the center of the circle was picked up using forceps), and one mL of a bead slurry was transplanted into this subcutaneous space. Figure 4 is a hematoxylin and eosin section of the collagen beads that were seeded with rat aortic smooth muscle cells. Note the highly porous structure of the beads (dark bands in the figure) and how the cells are growing within the beads after a six day period (lighter stained area). Figures 5A and 5B show the histological results after two weeks of implantation. Figure 5A is a histological section of collagen stain of the collagen beads implanted in a purse string subcutaneous space after two weeks implantation. Figure 5B is histological section of the collagen beads staining for Factor VIII which is an antigen expressed on the surface of endothelial cells. This is a marker for vascularization. Note the staining that has occurred within the collagen bead.

In a related experiment aortic smooth muscle cells were implanted in collagen beads. The histological results after two weeks of implantation are indicated by the dark stain of expressed α -actin. In Figure 7 there is shown the growth endothelial cells stained for Factor VIII on a collagen bead. The brown staining shows that cells are in the interstices of the bead, i.e., the cells migrated from the host.

EXAMPLE 4 Histological Observation of Two-week Subcutaneous Implants.

Many tissues and organs are organized as discrete tissue segments. These segments are generally arranged as islands of parenchymal or stromal cells that are surrounded by an intricate capillary bed. The size of these discrete tissue constructs is dictated by the metabolic need of the cells and diffusional distances for nutrients, gasses, and waste products to and from the cells.

The development of a three-dimensional vascular bed is imperative for the successful implantation of large tissue structures that have been created *ex vivo*. In order to develop a soft tissue that will replace the large mass of tissue that is removed from a patient following a lumpectomy, a strategy has been developed for the creation of a vascular bed to support cellular survival.

Alginate is a natural hydrogel that has previously been used for cell encapsulation and wound dressings. However, this material does not support cellular adhesion, as is required of any tissue engineering scaffold materials. Hence, a process was developed to couple the cellular adhesion tripeptide RGD to the alginate. The next task was to process this material into constructs of varying morphologies and therefore diffusional constraints.

Type I bovine collagen or sodium alginate was implanted into the subcutaneous spaces of Lewis rats. Type I bovine collagen was acquired as porous spheres (0.4 to 0.7 mm in diameter), whereas sodium alginate coupled with RGD tripeptide was fashioned either into rods, solid beads (1.0 to 3.0 mm in diameter), or porous beads (2.7 to 3.2 mm in diameter). The volume of each implanted material was one mL. The implants were harvested after two weeks implantation and characterized as to capsule formation, vascular ingrowth, tissue ingrowth, and inflammatory response. Table I outlines the experiment.

The Institutional Animal Care and Use Committee (IACUC) approved all animal procedures. A subcutaneous pocket was created on the back of a female Lewis rat using either a flank incision for the rods or a purse string suturing technique for the beads. The implants were harvested after 2 weeks and

histologically processed. Briefly, the animals were perfused with either Bouins fixative or Z-fix formalin to preserve the tissue biomaterial interface. After at least 24 hours in a formalin fixative, the tissue samples were carefully cut in half at the mid-line to preserve the tissue biomaterial interface. These small tissue blocks were then processed using a paraffin embedding method. Four micron sections were cut, and the tissues were stained with either hematoxylin and eosin, Masson's trichrome for the detection of collagen bundles, or stained with an antibody against Von willebrand's factor VIII (Dako, Cupertino, CA) for the detection of endothelial cells.

Three to six animals were evaluated for each implanted material type.

- At least two independent readers histologically scored the samples using 0 as a minimal and 4 as a maximal response. The histology samples were evaluated for vascular ingrowth, capsule formation, tissue ingrowth, and inflammation. The mean and standard deviations were calculated for all implanted materials, and an ANOVA analysis using Fisher's criteria was calculated using StatView 4.5 (Aacus, Berkeley, CA)

Table 1

Material	Dimensions	Number Implanted	Number of Recovered Implants (2 weeks)
Alginate-RGD Solid Rod	Implant volume = 1 mL Diameter = 1 cm and length = 1 cm	6	3*
Alginate-RGD Solid Bead	Implant volume = 1 mL Diameter = 1.5 – 3.0 mm	6	6
Alginate-RGD Porous Bead	Implant volume = mL Diameter = 2.5 – 3.0 mm	6	6
Cellex Type I Bovine Collagen Porous Beads	Implant volume = 1 mL Diameter = 400 – 700 mm	5	5**

Table 1: Experimental layout for biomaterials implanted into a Lewis rat model.

The number of samples analyzed for the implanted alginate rods was reduced due to the difficulty in preserving the tissue material interface during histological

5 processing.

*3 implants were lost during histological fixation due to the lack of tissue ingrowth into the material.

**One animal died post-transplant.

10

15 **Solid Rods and Beads:**

Alginate rods were formed by injecting a 1.2% alg-RGD solution into Spectra/Por dispodialyzers. These dialyzer tubes were 5ml volume and 10mm width, with a molecular weight cut-off of 300,000 and were pre-filled with sterile water. The water in the dispodialyzer case was poured out and replaced with sterile 1.5% CaCl_2 and then 3.3 mls of alg-RGD (in PBS) was placed inside the dialysis tube which was embedded in the case. The alginate-RGD was allowed to gel for one hour, and then the CaCl_2 was replaced with DMEM. At the time of surgery, the dispodialyzer tube membrane was cut away with small scissors, and the cylinder of alginate was placed into a petri dish. A ruler was placed under the dish, and the 25 alginate was cut into 1-cm rods with a sterile scalpel. The diameter was also 1 cm, making the implant approximately 1 ml, similar to the volume of the bead implants. For rod placement, an incision was made on the flank of the rat, blunt dissection used to form a pocket, the rod was placed inside the pocket, and the incision was stapled.

For solid beads, sodium alginate (Pronova, Lot #411-256-05, city/state) was covalently coupled with arginine, glycine, and aspartic acid tri-peptide (RGD). The alginate-RGD (alg-RGD) was mixed in calcium-free, magnesium-free phosphate buffered saline (Gibco/BRL) at a concentration of 2 gm/100 mL (2%). After thorough mixing, the alg-RGD was filtered using a 0.45 mm filter (Nalgene). The alg-RGD solution was then aseptically dropped into a sterile-filtered solution of 1.5% calcium chloride solution (Sigma Chemicals, St. Louis, MO) using a 60 mL syringe (Becton Dickinson) and a 30 gauge needle. A Harvard syringe pump (Harvard Industries) was used with a flow rate of 50 mL/hr.

10 Beads of approximately 1 to 3 mm in diameter were formed in this manner.

A subcutaneous pocket was created on the back of a female Lewis rat using a purse string suturing technique for the beads. The purse string was formed by using a 50-mL conical tube cap to mark a 39-mm circumference of the suturing plane, and then a 4.0 prolene suture was used to follow the outline of the circle. A 1.5 cm incision was made slightly off center in order to bluntly dissect the subcutaneous space before implanting a 1-mL bead slurry (either alginate or collagen beads), the incision was closed with Vicryl suture (Ethicon, Inc., Cincinnati, Ohio), and the prolene suture was tightened and tied to form a one mL subcutaneous pocket. All materials were pretreated overnight in a DMEM media containing (2%) fetal bovine serum.

15 20

Histologic analyses of tissue sections containing the solid alginate-RGD beads and rods were compared after 2 weeks of implantation. A fibrous capsule had formed around the rods and the smaller bead implants with no discernible tissue ingrowth into the material. This lack of tissue ingrowth into the alginate rods made it difficult to maintain the tissue-biomaterial interface during histological processing. There was a minimal presence of giant and polymorphonuclear cells (PMNs) in both of these implant sites. At high magnification, the capsule that formed around the alginate-RGD beads could be seen. The capsule appeared to be highly vascularized. An immunohistochemical

25

section identified the presence of Factor VIII throughout the capsule region. (This marker is used to identify endothelial cells.)

The ability to form a highly vascularized capsule around the beads and rods is an important factor for the delivery of nutrients. However, the size of these constructs (greater than 1.5 mm) is an order of magnitude greater than the diffusional distance of nutrients and oxygen (0.15 mm).; therefore, cells delivered in these materials would not receive an adequate nutrient supply.

Porous Collagen Beads:

A porous bovine Type I collagen bead was chosen to determine if a three-dimensional vascular bed could be established with the requisite dimensions to support transplanted cells. Type I collagen beads were purchased from Cellex (Minnesota). The beads were glutaraldehyde fixed, with an average diameter of 0.4 - 0.7 mm and pore size ranging from 0.05 to 1 mm. The beads were highly porous, ranging in diameter from 0.4 to 0.7 mm, with a pore size range of 0.05 to 0.1 mm, and they promoted cellular attachment. Type I collagen beads were implanted on the back of female Lewis rats using the purse string implant technique described above. Histological examination of a section of the implanted Type I porous collagen beads showed that, unlike the alginate beads, there was a cellular ingrowth within the bead structure. However, there was also an increased presence of giant cells and PMNs throughout the tissue construct. Upon staining for Factor VIII within these transplanted areas, it was observed that vascular endothelial cells are located throughout the implanted area.

25

Macroporous Alginate-RGD Beads

Mimicking this microarchitecture, a macroporous alginate-RGD bead was developed with a diameter range between 2.7 and 3.2 mm, and an undetermined pore size range. Porous alginate-RGD beads were made using 3% alginate-RGD

mixed with a 2 M sodium bicarbonate and a 1.5% BSA solution (1:1:1). After approximately 15 minutes of stirring on a stir plate at medium rotation, the alginate-RGD became foamy and thick. This foamy material was located into a 10 mL syringe and dripped into a calcium chloride solution (0.5 M calcium chloride solution mixed in glacial acetic acid at 9:1 volume ratio) at a rate of approximately 1 bead per second. The beads were rinsed in sterile double distilled water, and the beads were placed in 700 ml of 0.33 M calcium chloride solution. This bead mixture was then placed on a Buchi (Buchi, Switzerland) rotavapor, and vacuum was applied until the beads sank to the bottom of the vessel. The beads were sterilized using gamma irradiation. The porous alginate-RGD beads were implanted on the back of female Lewis rats using the purse string implant technique described above. Histological cross-section of a porous alginate-RGD bead after 2 weeks implantation showed that a vascular bed is present throughout the bead implant.

15 **Summary:**

 Histological sections of the implanted alginate-RGD and Type I collagen beads were graded according to capsule formation, presence of vasculature within the implant site, tissue ingrowth, and the inflammatory response, and the results are presented in bar graph format in Figure 8. A scale of 0 to 4 was assigned to each of these observations, with a 0 being a minimal and a 4 being a maximal quantity. As indicated in Figure 8, both implanted materials provided a mechanism for the development of a vascular bed. However, the solid alginate beads did not permit tissue ingrowth into the material, whereas the Type I collagen beads and the porous alginate-RGD beads supported tissue ingrowth throughout the implant site.

25 The alginate-RGD beads had a minimal presence of giant cells and PMNs, while the Type I collagen beads showed a moderate to severe inflammatory reaction. The inflammatory response to the Type I collagen beads may have been due to the glutaraldehyde fixation method used to covalently modify the material.

The animal model employed in this experiment provided the ability to test whether a vascular bed could be established in a large defect. On average, the distance from the host vascular bed to the center of the bead implant was 2.0 mm post-histological processing. The ability to create a three-dimensional vascular bed with this thickness will allow for soft tissue reconstruction. It is also feasible through multiple step implants of a similar thickness to serially increase tissue volumes. This approach could be used to develop the larger tissue masses needed for breast reconstruction following a lumpectomy.

10 EXAMPLE 5 Histological Observation of Subcutaneous Implants at 2, 4, and 8 Weeks.

Macroporous alginate-RGD beads were prepared and implanted as described in Example 4, and histological observations were taken as described at 2, 4, and 8 weeks after the implants. The results are shown in Figures 9-13. The porous alginate-RGD beads allowed tissue ingrowth with the presence of a vascular bed throughout the implanted material. Figure 13 shows that the maximum thickness of the surrounding capsule (corresponding to scar tissue) occurs at two weeks (on the side of the implant facing muscle) or four weeks (on the side of the implant facing skin) and decreases by eight weeks.

20 Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

In the specification, there has been set forth a preferred embodiment of the invention and, although specific terms are employed, the terms are used in a generic and descriptive sense only and not for purpose of limitation, the scope of the invention being set forth in the following claims.

All publications and patent applications mentioned in the specification are indicative of the level of those skilled in the art to which this

invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Likewise, the parent application of this application is incorporated herein by

5 reference.

THAT WHICH IS CLAIMED IS:

1. A method of providing a vascularized three-dimensional tissue in a living subject comprising delivering into an area of interest in said living subject a plurality of discrete, macroporous constructs, said constructs comprising
 - 5 (a) a biocompatible material having a porous structure; and
 - (b) cells disposed in said biocompatible material within 400 μm from at least one surface of said material, wherein an increase in vascularization occurs in said area of interest subsequent to delivery of said plurality of constructs.
2. The method of providing a vascularized three-dimensional tissue in
10 accordance with Claim 1, wherein said biocompatible material is a bioresorbable material.
3. The method of providing a vascularized three-dimensional tissue in accordance with Claim 1, wherein said biocompatible material is a non-resorbable material.
- 15 4. The method of providing a vascularized three-dimensional tissue in accordance with Claim 2, wherein said bioresorbable material is selected from the group consisting of polyesters, polyethylene glycols, and hydrogels.
5. The method of providing a vascularized three-dimensional tissue in accordance with Claim 3, wherein said non-resorbable biocompatible material is
20 selected from the group consisting of polytetrafluoroethylenes, polycarbonates, polyethylene, and nylons.
6. The method of providing a vascularized three-dimensional tissue in accordance with Claim 2, wherein said bioresorbable material is selected from the group consisting of polyvinyl alcohols, polyacrylamides, polyamides, polyacrylates,
25 polyesters, polymethacrylates, polyurethanes, polyphosphazene, copolymers lactic acid and glycolic acid, copolymers of lysine and lactic acid, and copolymers of lysine-RGD and lactic acid.

7. The method of providing a vascularized three-dimensional tissue in accordance with Claim 1, wherein said biocompatible material is selected from the group consisting of hydrogels and naturally occurring polymers.

8. The method of providing a vascularized three-dimensional tissue in accordance with Claim 7, wherein said biocompatible material is alginate.

9. The method of providing a vascularized three-dimensional tissue in accordance with Claim 7, wherein said biocompatible material is a collagen.

10. The method of providing a vascularized three-dimensional tissue in accordance with Claim 1, said method further comprising delivering into said area of interest a signal for modifying cell adhesion, growth, or migration.

11. The method of providing a vascularized three-dimensional tissue in accordance with Claim 10, wherein said signal is selected from growth factors, hormones, extracellular matrix proteins, and cell adhesion peptides.

12. The method of providing a vascularized three-dimensional tissue in accordance with Claim 11, wherein said growth factor is selected from the group consisting of epithelial growth factor, acidic or basic fibroblast growth factor, vascular endothelial growth factor, hepatocyte growth factor, heparin binding growth factor, transforming growth factor, nerve growth factor, muscle morphogenic factor, and platelet derived growth factor.

13. The method of providing a vascularized three-dimensional tissue in accordance with Claim 10, wherein said signal is a cell adhesion peptide selected from the group consisting of tri-peptide RGD and YIGSR.

14. The method of providing a vascularized three-dimensional tissue in accordance with Claim 10, wherein said signal is a collagen.

15. The method of providing a vascularized three-dimensional tissue in accordance with Claim 10, wherein the signal is covalently coupled to the biocompatible material.

16. The method of providing a vascularized three-dimensional tissue in a living subject according to claim 1, wherein the plurality of discrete, macroporous constructs has a maximum particle size of about 5 mm.

17. The method of providing a vascularized three-dimensional tissue in a
5 living subject according to claim 1, wherein each discrete, macroporous construct has a center which is no more than 400 μm from each outer surface of said construct.

18. The method of providing a vascularized three-dimensional tissue in a living subject according to claim 1, wherein said plurality of discrete, macroporous constructs have pores with pore size between 25 μm and 150 μm .

10 19. The method of providing a vascularized three-dimensional tissue in a living subject according to claim 1, wherein delivery of said plurality of constructs is by injection.

20. The method of providing a vascularized three-dimensional tissue in a living subject according to claim 1, wherein said plurality of constructs is delivered
15 to said area of interest in said living subject in a biocompatible pouch having an arteriole and venule connection from outside the pouch into the pouch.

21. The method of providing a vascularized three-dimensional tissue in accordance with Claim 1, further comprising growing said cells before said delivering step.

20 22. The method of providing a vascularized three-dimensional tissue in accordance with Claim 1, wherein said cells are smooth muscle cells.

23. The method of providing a vascularized three-dimensional tissue in accordance with Claim 1, wherein said cells are selected from the group consisting of smooth muscle cells, endothelial cells, hepatocytes, adipocytes, fibroblasts, renal
25 tubular cells, Sertoli cells, thyroid cells, islet cells, skeletal muscle cells, adrenal cells, neural cells, cardiac muscle cells, chondrocytes, osteocytes, stem cells, and the like.

24. The method of providing a vascularized three-dimensional tissue in accordance with Claim 1, wherein said cells are selected from the group consisting of allogeneic, autologous and xenogeneic cells.

25. The method of providing a vascularized three-dimensional tissue in accordance with Claim 1, wherein said living subject is human.

26. A method of providing a vascularized three-dimensional tissue in a living subject comprising delivering into an area of interest in said living subject a plurality of discrete, macroporous constructs, said constructs comprising.

- (a) a biocompatible material having a porous structure;
 - 10 (b) cells disposed in said biocompatible material within 400 μm from at least one surface of said material; and
 - (c) a signal for modifying cell adhesion, growth, or migration,
- wherein an increase in vascularization occurs in said area of interest subsequent to delivery of said plurality of constructs.

15 27. The method of providing a vascularized three-dimensional tissue in accordance with Claim 26, wherein said biocompatible material is selected from the group consisting of alginates and type I collagen.

28. The method of providing a vascularized three-dimensional tissue in accordance with Claim 26, wherein said signal is a cell adhesion peptide covalently
20 linked to said biocompatible material.

29. A method of repairing a soft tissue defect in a living subject comprising delivering into a living subject having a soft tissue defect a plurality of discrete, macroporous constructs, said constructs comprising

- (a) a biocompatible material having a porous structure;
- 25 (b) cells obtained from said living subject and selected from the group consisting of smooth muscle cells, endothelial cells, mesenchymal cells, fibroblasts, chondrocytes, and adipocytes,

wherein said cells are disposed in said biocompatible material within 400 μm from at least one surface of said material, and delivery of said plurality of constructs is sufficient to at least partially correct said soft tissue defect.

30. The method of repairing a soft tissue defect in accordance with Claim
5 29, wherein said a plurality of discrete, macroporous constructs further comprise a signal for modifying cell adhesion, growth, or migration.

31. The method of repairing a soft tissue defect in accordance with Claim
29 or Claim 30, wherein said living subject is a human and said soft tissue defect is a deficiency of breast tissue.

10 32. A method of providing a vascularized three-dimensional tissue in a living subject comprising

(a) delivering into said area of interest a biocompatible material supporting the formation of a vasculature bed *in vivo*, wherein a vasculature bed develops in said area of interest; and

15 (b) delivering cells adjacent to said vasculature bed,
wherein a vascularized three-dimensional tissue develops in said area of interest subsequent to delivery of said cells.

33. The method of providing a vascularized three-dimensional tissue in a living subject according to claim 32, wherein said cells are contained in a plurality
20 of discrete, macroporous constructs, said constructs comprising:

- (i) a biocompatible material having a porous structure; and
- (ii) cells disposed in said biocompatible material within 400 μm from at least one surface of said material.

34. The method of providing a vascularized three-dimensional tissue in a
25 living subject according to claim 1, wherein said constructs are coated with a material that will promote vascular endothelial growth.

35. The method of providing a vascularized three-dimensional tissue construct in accordance with Claim 34, wherein said coating is selected from the

group consisting of fibronectin, vitronectin, collagens, polylysine, laminins, polypeptides derived from these extra-cellular matrices, and hydrogels.

36. A composition for providing a vascularized three-dimensional tissue in a living subject comprising a plurality of discrete, macroporous constructs, said
5 constructs comprising

- (a) biocompatible material having a porous structure; and
- (b) cells disposed in said biocompatible material within 400 μm from one at least surface of said material, wherein an increase in vascularization occurs in said area of interest subsequent to delivery of said plurality of constructs.

10 37. The composition of claim 36, further comprising a signal for modifying cell adhesion growth, or migration.

38. Use of biocompatible material in the preparation of a composition for providing a vascularized three-dimensional tissue in a living subject by delivering into an area of interest in said living subject a plurality of discrete, macroporous
15 constructs, said plurality of discrete, macroporous constructs comprising

- (a) a biocompatible material having a porous structure; and
- (b) cells disposed in said biocompatible material within 400 μm from at least one surface of said material, wherein an increase in vascularization occurs in said area of interest subsequent to delivery of said plurality of constructs.

20 39. A kit for providing a vascularized three-dimensional tissue in a living subject by delivering into an area of interest in said living subject a plurality of discrete, macroporous constructs, said kit comprising

- (a) a biocompatible material supporting the formation of a vasculature bed *in vivo*, wherein delivery of said biocompatible material into an area of interest in said
25 living subject induces development of a vasculature bed in said area of interest; and
- (b) a plurality of discrete, macroporous constructs, said constructs comprising:
 - (i) a biocompatible material having a porous structure; and
 - (ii) cells disposed in said biocompatible material within 400 μm from at least one source of said material,

wherein delivering of said plurality of discrete, macroporous constructs adjacent to said vasculature bed induces a vasculaturized three-dimensional tissue to develop in said area of interest subsequent to delivery of said plurality of discrete, macroporous constructs.



FIG. 1A



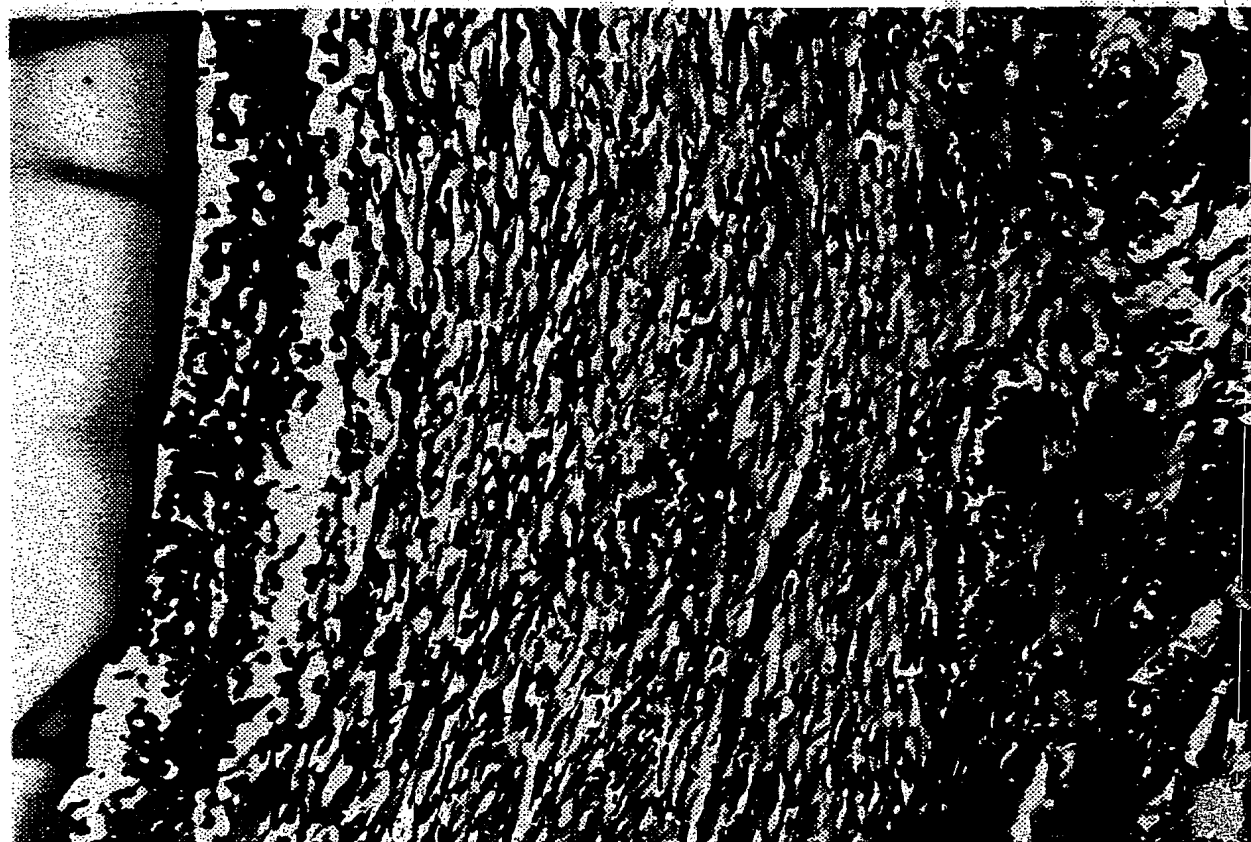
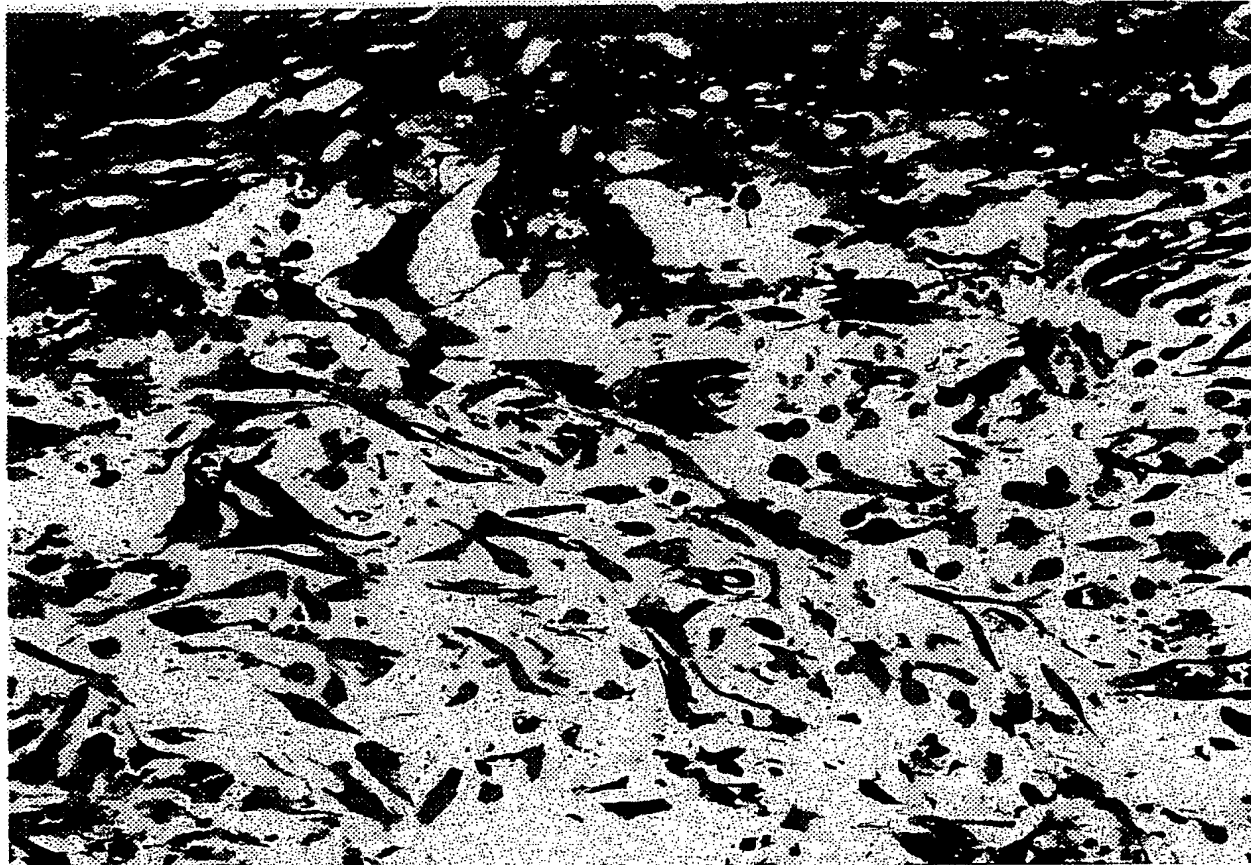


FIG. 1B



FIG. 2A

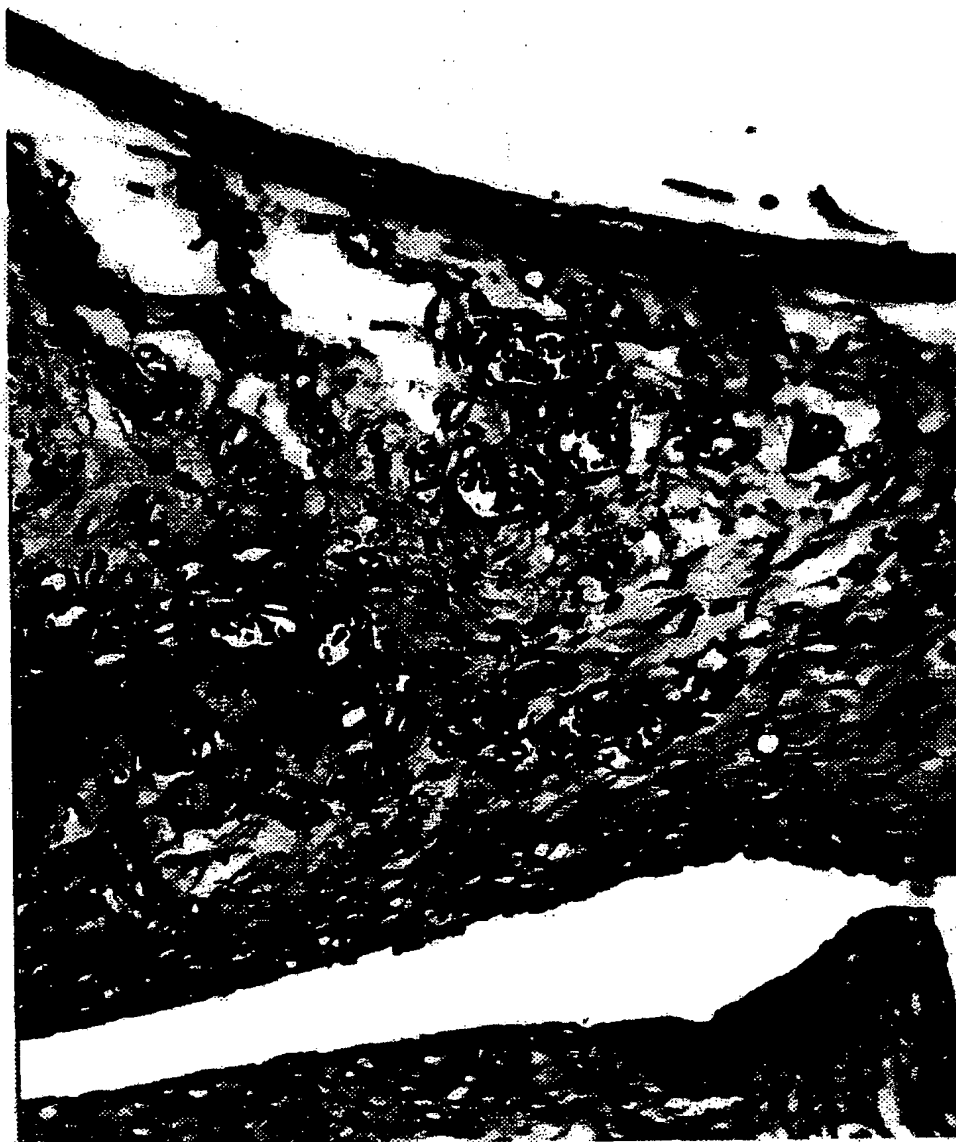


FIG. 2B

SUBSTITUTE SHEET (RULE 26)



FIG. 3

SUBSTITUTE SHEET (RULE 26)

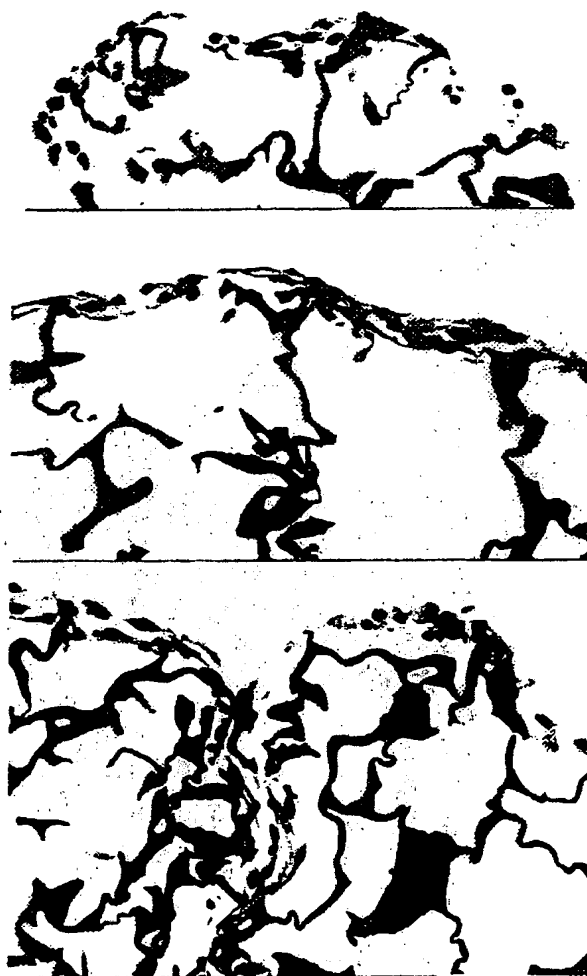
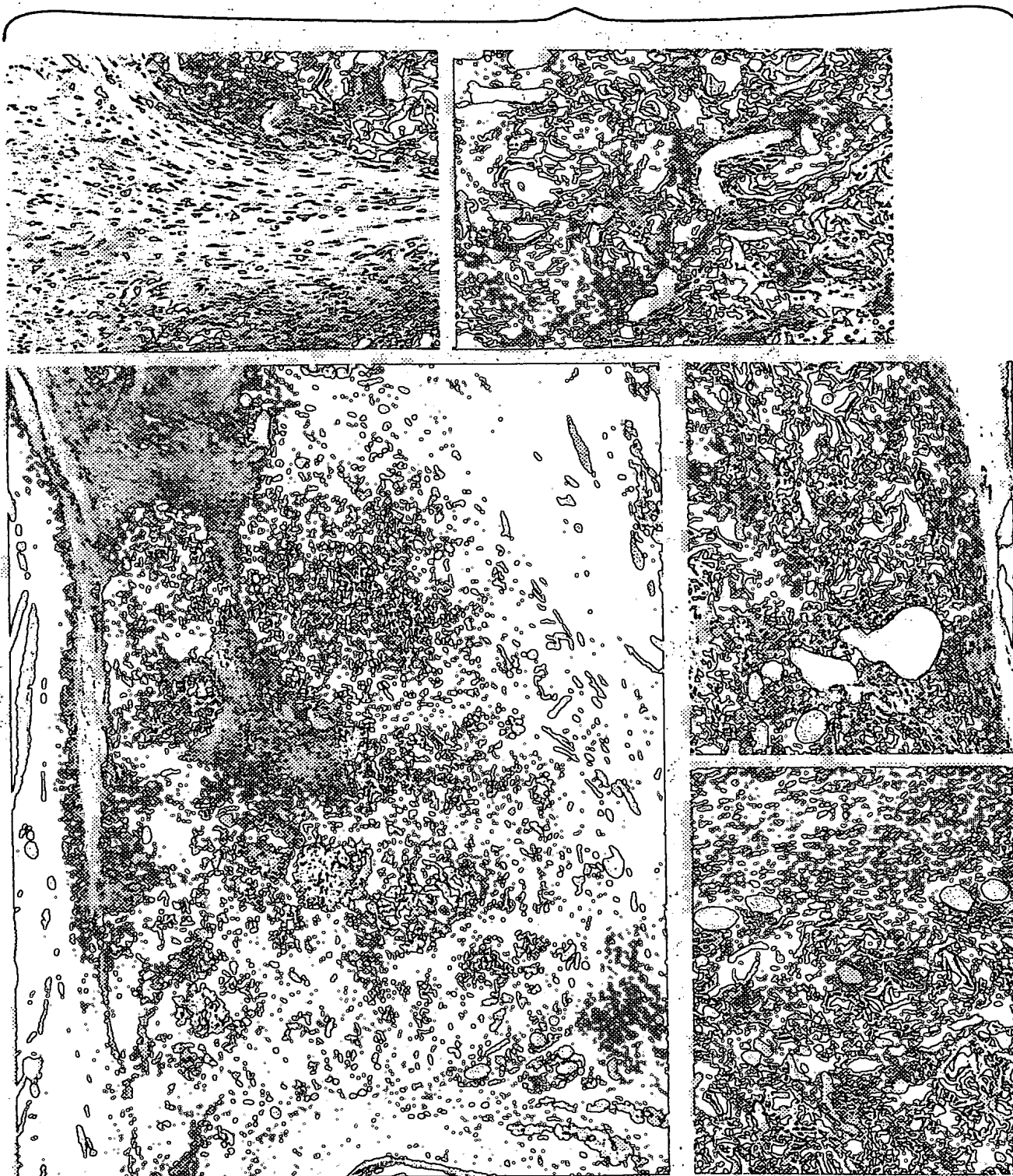


FIG. 4

FIG. 5A



SUBSTITUTE SHEET (RULE 26)



FIG. 6.

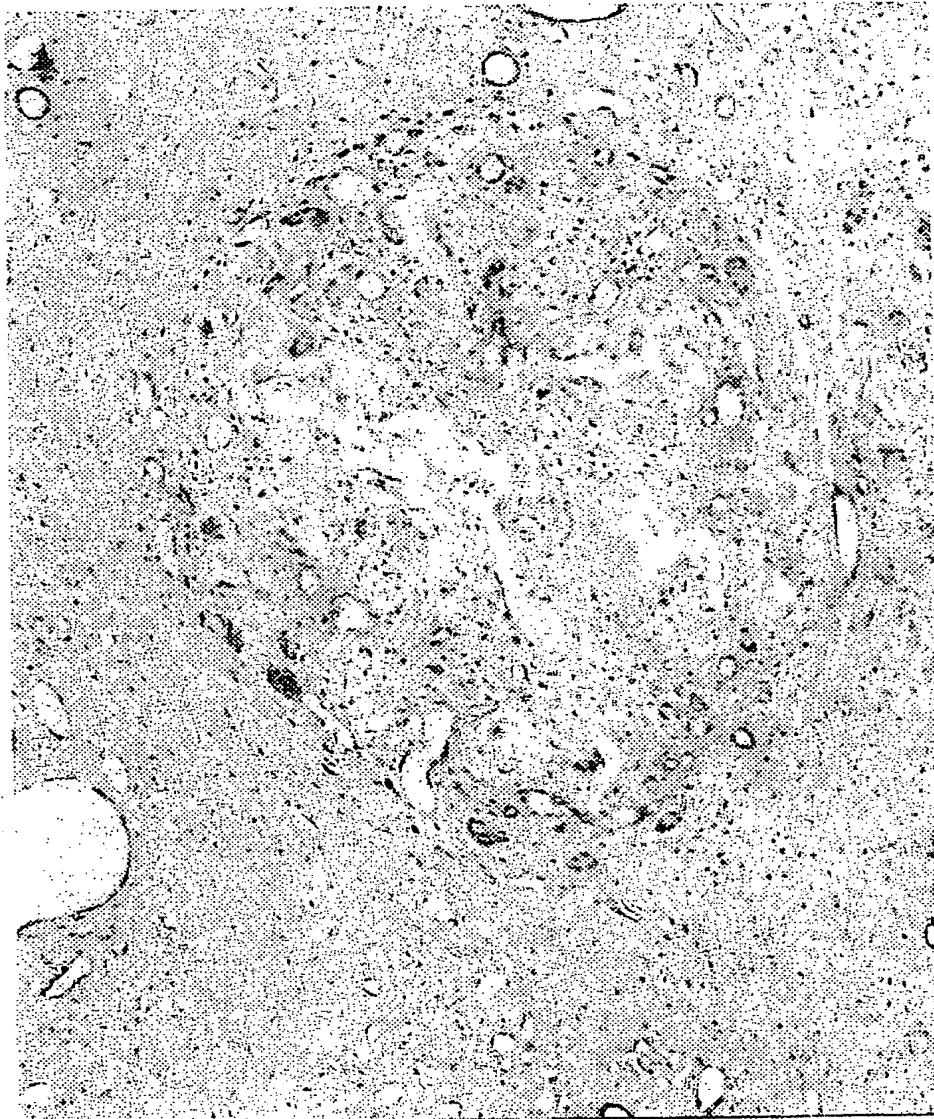


FIG. 7

SUBSTITUTE SHEET (RULE 26)

10/15

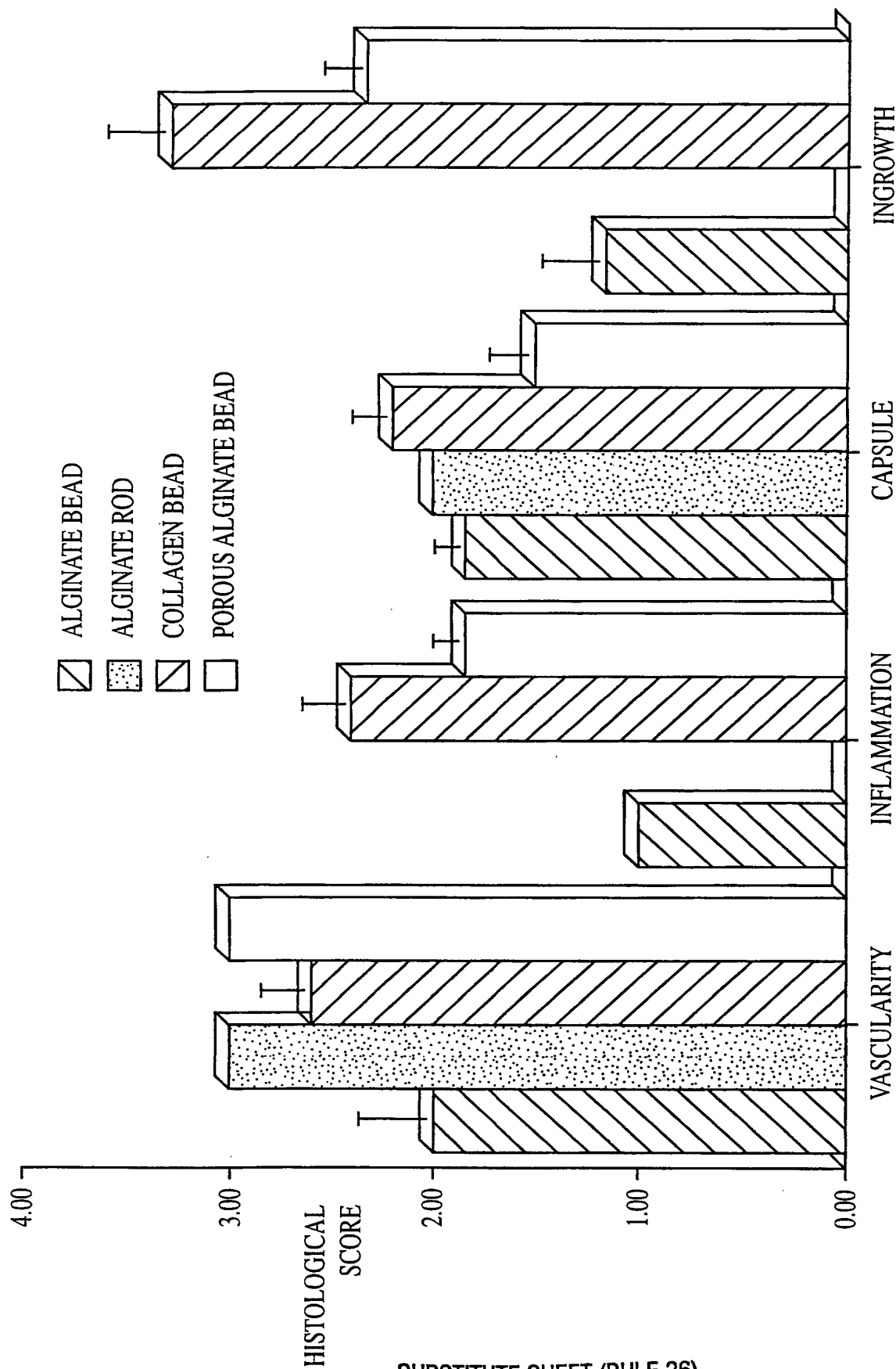


FIG. 8

11/15

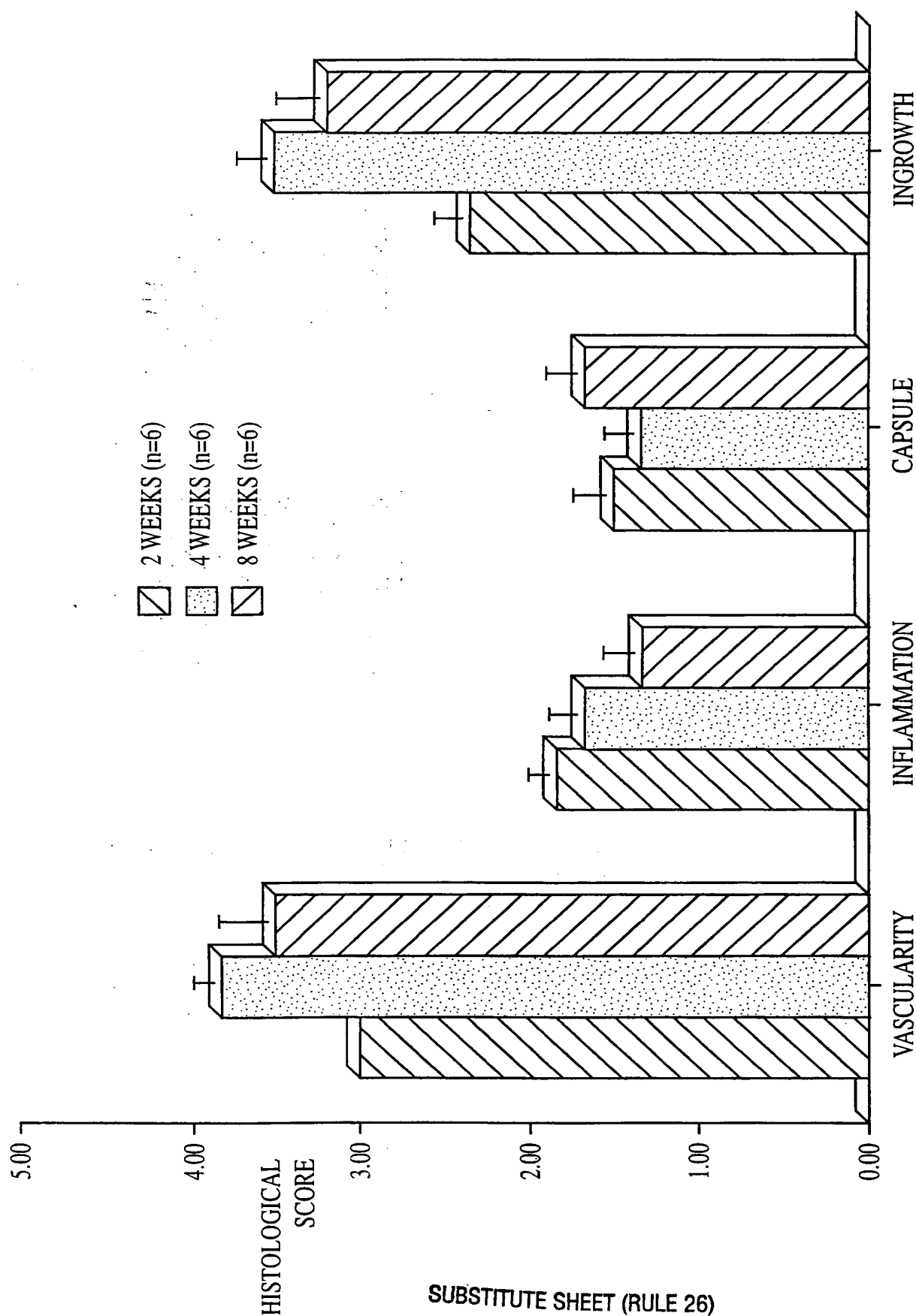
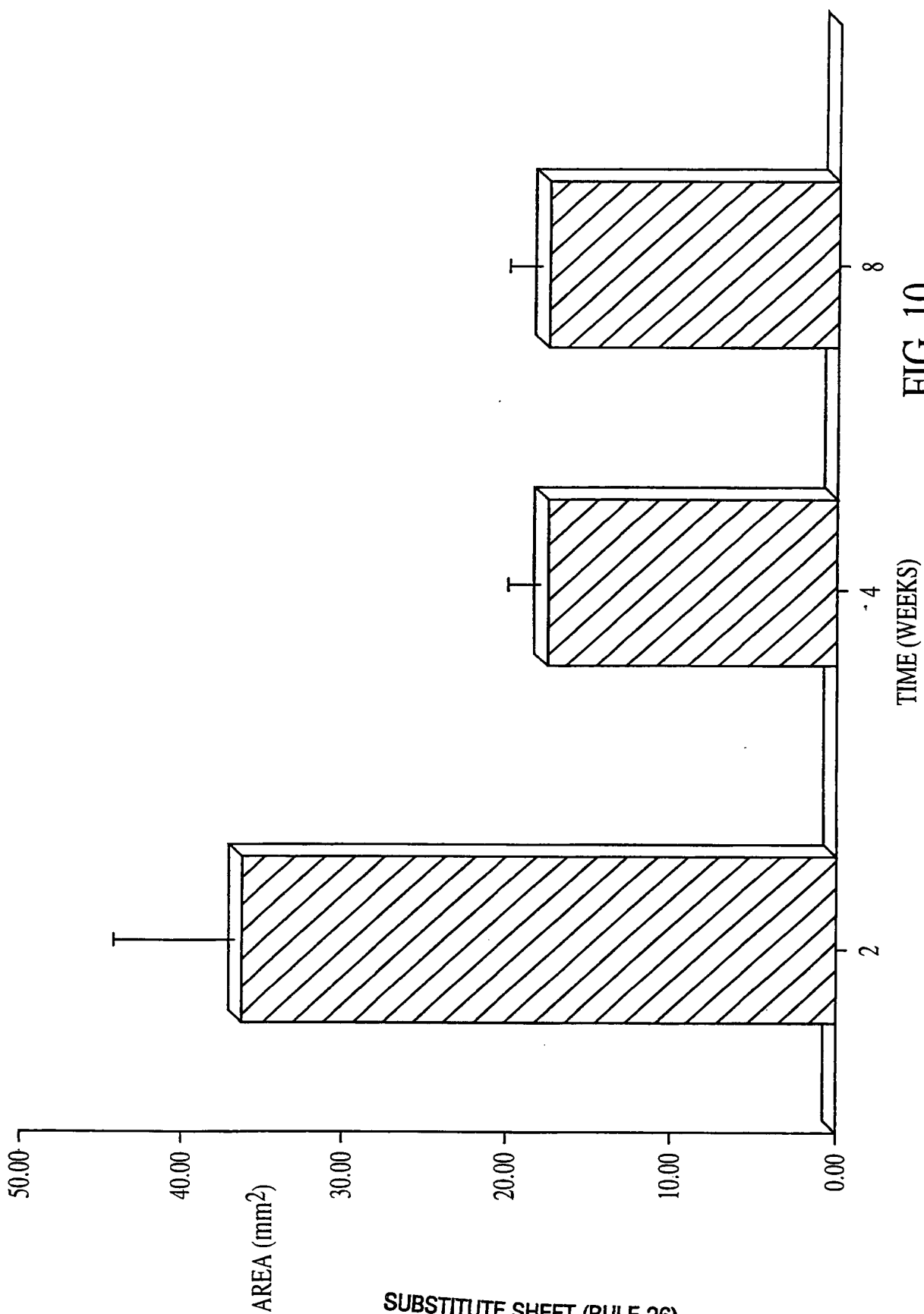


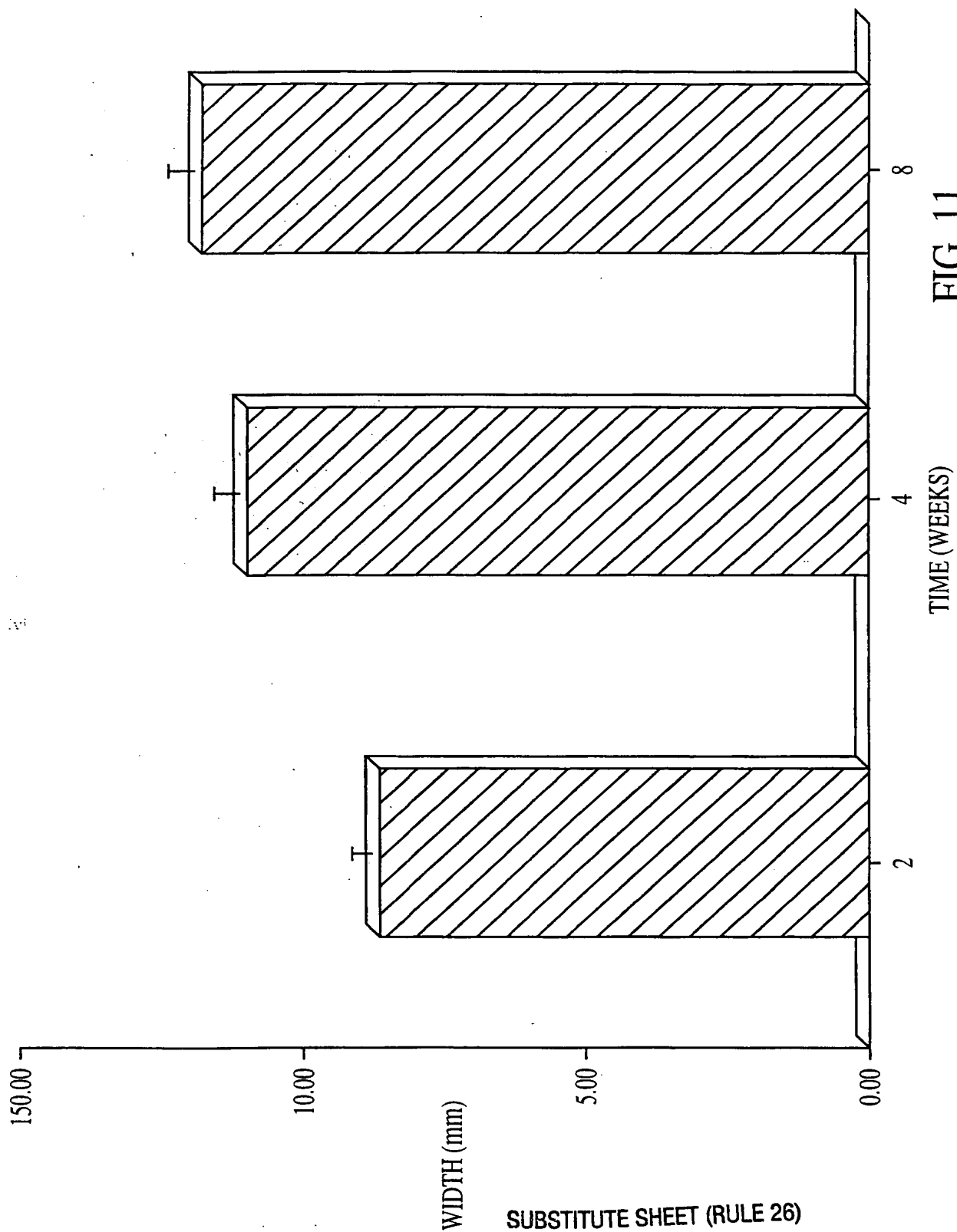
FIG. 9

12/15

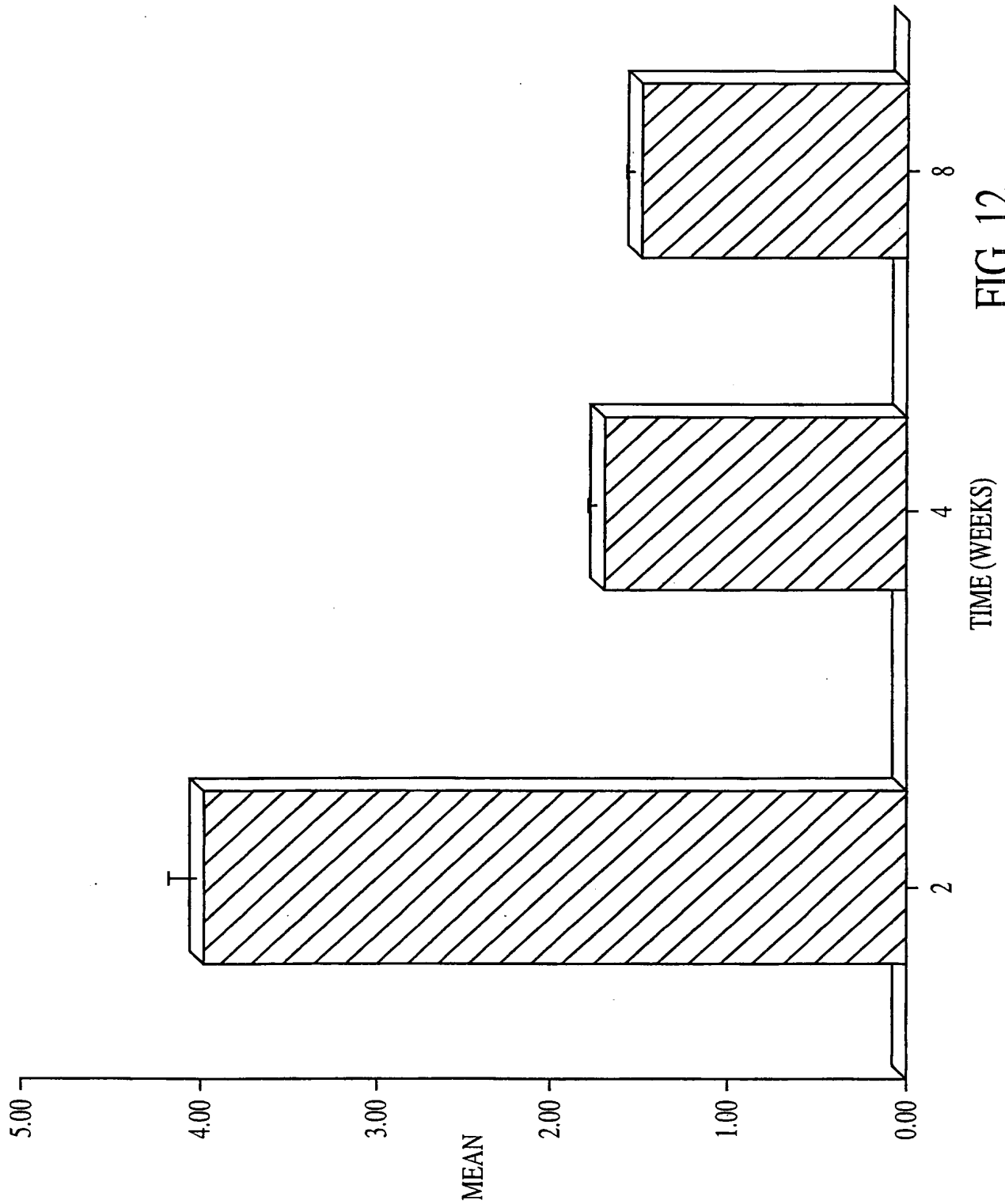


SUBSTITUTE SHEET (RULE 26)

13/15



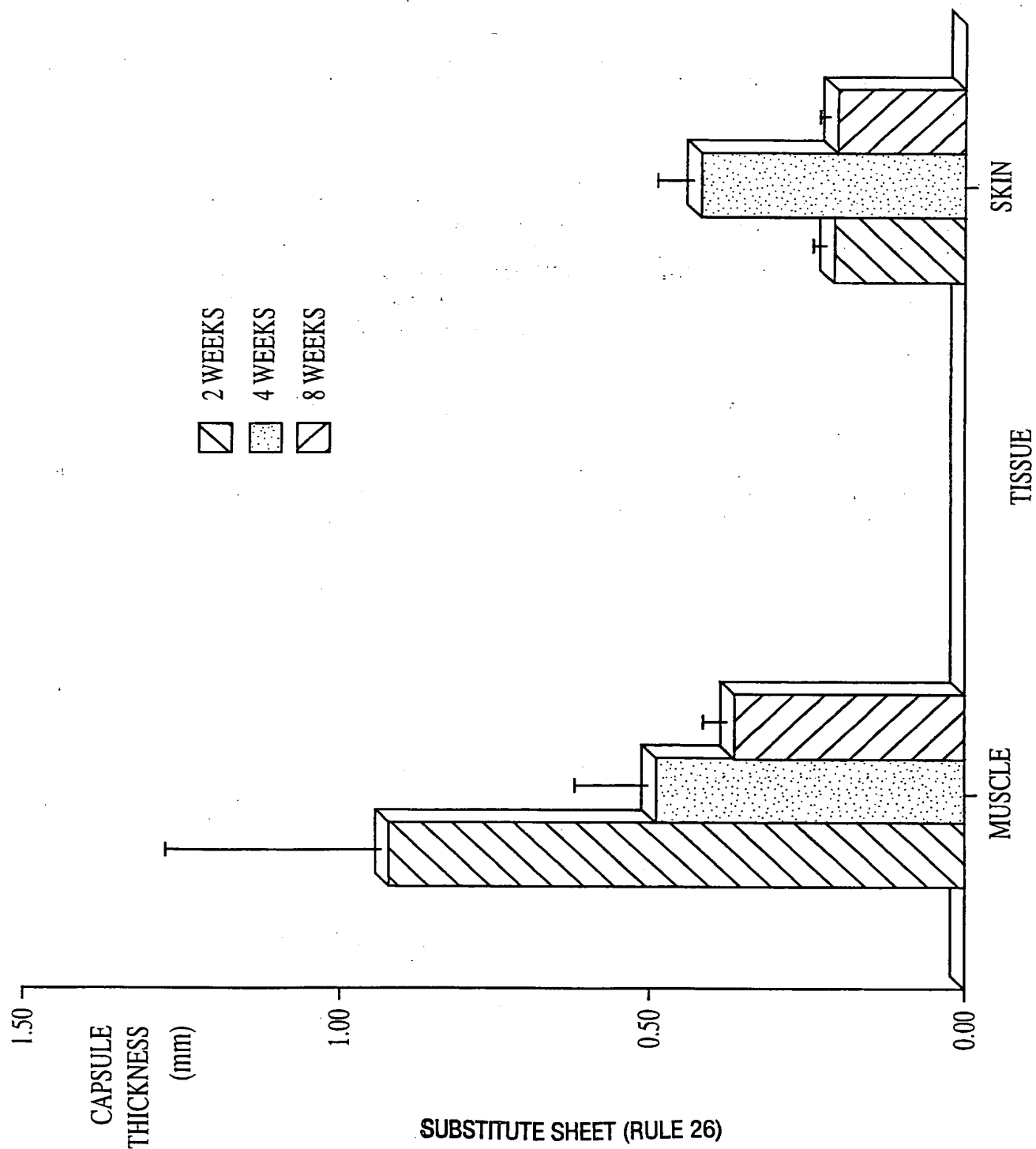
14/15



SUBSTITUTE SHEET (RULE 26)

15/15

FIG. 13



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/07816

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A01N 1/02; C12P 19/34; C12M 3/00

US CL : 435/ 1.1, 93.1, 283.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/ 1.1, 93.1, 283.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
NONEElectronic data base consulted during the international search (name of data base and, where practicable, search terms used)
APS, caplus, biosis, medline

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BARRERA et al. Synthesis and RGD peptide modification of a new biodegradable copolymer: poly(lactic acid-co-lysine). 1993. J. Am. Chem.Soc. 1993, Vol. 115, No. 23. pages 11010-11011, see entire article.	1-39
Y	US 5,716,404 A (VACANTI et al) 10 February 1998, col. 4, line 26.	1-39
A	GRANDE et al. Evaluation of matrix scaffolds for tissue engineering of articular cartilage grafts. J. Biomed. Materials Res. 1997, Vol. 34, pages 211-220.	1-39



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
B earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A*	document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

26 MAY 1999

Date of mailing of the international search report

09 JUL 1999

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

MICHAEL C. WILSON

Telephone No. (703) 308-0196